PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of Docket No: 00766.000044

Yoshiki SASAI, *et al.*

Appln. No.: 09/855,587 Group Art Unit: 1632

Filed: May 16, 2001 Examiner: SGAGIAS, MAGDALENE K.

For: NOVEL DIFFERENTIATION INDUCING PROCESS OF EMBRYONIC STEM CELL TO ECTODERMAL CELL AND ITS USE

DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir/Madam:

I, Hiromasa MIYAJI, Ph.D., of 5-41-5, Matsubara, Setagaya-ku, Tokyo, 156-0043 Japan do hereby declare as follows,

I graduated from the Department of Agriculture of TOKYO University in March, 1981, and got the degree of Ph.D. from TOKYO University in 1991. Since April, 1983, I have been employed by Kyowa Hakko Kogyo Co., Ltd. I was assigned to Tokyo Research Laboratories of the company in July, 1983, and was engaged in research and development of pharmaceutical products. From October, 1993 through March, 1995, I conducted insulin-signaling related research at Joslin Diabetes Center, Harvard Medical School in the United States, holding my title and position at Kyowa Hakko Kogyo Co., Ltd. Since April, 1995, as a Senior Researcher, I was engaged in molecular cloning of pharmaceutically useful targets at Tokyo Research Laboratories. Since January, 1996, as a Senior Researcher, I was engaged in genome-based drug discovery at Pharmaceutical Research Institute of Kyowa Hakko Kogyo Co., Ltd. Since July, 2003, as a Senior Researcher, I have been engaged in the research of regenerative medicine at Tokyo Research Laboratories of Kyowa Hakko Kogyo Co., Ltd, which are currently named BioFrontier Laboratories.

I have reviewed the prosecution of the present application.

The following experimentation was conducted by me or my direct supervision.

EXPERIMENTATION

Analysis of expression of KM1310 antigen molecule in PA-6, OP9, NIH3T3 and MEF cells:

As stroma cells, PA-6, OP9 and NIH3T3, the cells described in the specification of the present application were used. Mouse embryonic fibroblast cells (MEF) were purchased from ATCC.

PA-6 was cultured in a medium in which α-MEM (manufactured by Gibco) was supplemented with 10% fetal calf serum and 100 U/mL penicillin-100 μg/mL streptomycin (manufactured by Gibco) under conditions at 37°C and 5% CO_2 in a CO_2 incubator.

OP9 was cultured in a medium in which MEM (manufactured by Gibco) was supplemented with 10% fetal calf serum and 100 U/mL penicillin-100 μg/mL streptomycin under conditions at 37°C and 5% CO_2 in a CO_2 incubator.

NIH3T3 was cultured in a medium in which DMEM (manufactured by Gibco) was supplemented with 10% fetal calf serum, 0.1 mmol/L MEM Non-Essential Amino Acids Solution (manufactured by Gibco) and 100 U/mL penicillin-100 μg/mL streptomycin under conditions at 37°C and 5% CO_2 in a CO_2 incubator.

MEF was cultured in a medium in which DMEM (manufactured by Gibco) was supplemented with 15% fetal calf serum, 1.5 g/L sodium bicarbonate solution (manufactured by Gibco) and 100 U/mL penicillin-100 μg/mL streptomycin under conditions at 37°C and 5% CO_2 in a CO_2 incubator.

After the cell density reached almost confluence, each cells were washed in phosphate-buffered saline [hereinafter referred to as "PBS(-)"]. After TrypLE Select (manufactured by Invitrogen) was added thereto at 37°C for 2 minutes to remove the cells, the reaction was stopped by adding a PBS(-) solution containing 5% serum. The cells were recovered by centrifugation at 1000 rpm for 5 minutes, suspended at 1×10^6 cells in 100 μL of a monoclonal antibody KM1310 solution prepared from a culture supernatant of hybridoma FERM BP-7573 as a primary antibody, and allowed to react at 4°C for 30 minutes. Separately, the cells were suspended at 1×10^6 cells in 100 μL

of a PBS(-) solution containing 5% serum, rat IgM (manufactured by Becton and Dickinson) was added thereto at 5% concentration, and the cells were allowed to react at 4°C for 30 minutes as the negative control. After washing the cells in a PBS(-) solution containing 5% serum, the cells were recovered by centrifugation at 4500 rpm for 1 minute and suspended in a PBS(-) solution containing 5% serum. As the second antibody, FITC-labeled anti-rat Ig antibody (manufactured by Becton and Dickinson) was added to give the final concentration 5% and the mixture was incubated at 4°C for 30 minutes. After washing the cells three times in a PBS(-) solution containing 5% serum, the cells were suspended in 1 mL of a PBS(-) solution containing 5% serum and analyzed by flow cytometory (manufactured by Becton and Dickinson, FACS Aria). Dead cells were removed by adding 2 μg of propidium iodide (manufactured by Invitrogen).

In each cells, the ratio of the mean fluorescence intensity (MFI) of the KM1310 treated group to that of the negative control group was calculated to use as the ratio of the KM1310 antigen expression level. As shown in the following table, the MFI ratio was 1.30 to 1.72 in PA-6, OP9 and NIH3T3, indicating that these cells could express KM1310 antigen. On the other hand, the MFI ratio in MEF was 0.99, indicating that MEF cell line could not express KM1310 antigen molecule.

From these results, it was found that PA-6, OP9 and NIH3T3 which are stroma cells used in the present invention were recognized by a monoclonal antibody KM1310 produced from hybridoma FERM BP-7573 and that mouse embryonic fibroblast cells were not recognized by the antibody.

TABLE

Cells	PA-6	OP9	NIH3T3	MEF
MFI Ratio	1.57	1.30	1.72	0.99

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date : Nov. 6, 2007 Name : /Hiromasa Miyaji/
Hiromasa MIYAJI, Ph.D.